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Identification of an *Arabidopsis thaliana* protein that binds to tomato mosaic virus genomic RNA and inhibits its multiplication

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ABSTRACT

The genomic RNAs of positive-strand RNA viruses carry RNA elements that play positive, or in some cases, negative roles in virus multiplication by interacting with viral and cellular proteins. In this study, we purified *Arabidopsis thaliana* proteins that specifically bind to 5' or 3' terminal regions of tomato mosaic virus (ToMV) genomic RNA, which contain important regulatory elements for translation and RNA replication, and identified these proteins by mass spectrometry analyses. One of these host proteins, named BTR1, harbored three heterogeneous nuclear ribonucleoprotein K-homology RNA-binding domains and preferentially bound to RNA fragments that contained a sequence around the initiation codon of the 130K and 180K replication protein genes. The knockout and overexpression of *BTR1* specifically enhanced and inhibited, respectively, ToMV multiplication in inoculated *A. thaliana* leaves, while such effect was hardly detectable in protoplasts. These results suggest that BTR1 negatively regulates the local spread of ToMV.

Introduction

After entry into the host cell, the genomic RNA of a positive-strand RNA virus is released from virions into the cytoplasm, where the genomic RNA is translated to produce the replication proteins and other viral gene products. Then, the genomic RNA is specifically recognized, recruited to the cytoplasmic surfaces of organellar membranes to form the replication complex, and utilized as the template of negative-strand RNA synthesis (Ahlquist, 2006). The replicated genomic RNA assembles with the structural proteins into virions. For translation, replication, and encapsidation of the genomic RNA, specific RNA sequences or structures in the genomic RNA play important roles by interacting with each other (Miller and White, 2006) and/or with viral and/or cellular proteins (Martinez-Salas et al., 2008; White and Nagy, 2004). Such RNA elements often reside in the 5' and 3' untranslated regions (UTRs) (Dreher and Miller, 2006; Sullivan and Ahlquist, 1997), but some exist in the coding regions (McKnight and Lemon, 1998; Zimmern, 1977). Some specific interactions between viral genomic RNA and cellular proteins have negative effects on the viral life cycle (Paranjape and Harris, 2007; Zhu et al., 2007).

Tomato mosaic virus (ToMV) is a positive-strand RNA virus that belongs to the genus *Tobamovirus*. The genome of ToMV is a 5' m^7Gppp(G)-capped monopartite RNA of 6384 nucleotides (nts), and encodes at least four proteins: a 130-kDa (130K) protein and its readthrough product of 180 kDa (180K protein), a 30-kDa protein, and a coat protein (CP). The 130K and 180K proteins are synthesized from the genomic RNA and involved in viral RNA replication, suppression of RNA silencing, and cell-to-cell movement. The 30-kDa protein is required for viral cell-to-cell movement. The 30-kDa protein and CP are synthesized by translation of the respective subgenomic RNAs (Ishikawa and Okada, 2004; Kubota et al., 2003).

ToMV genomic RNA harbors 5' and 3' UTRs of 71 nts and 202 nts, respectively. Because some modifications in the 5' or 3' UTRs deleteriously affect infectivity (Takamatsu et al., 1990; 1991), these UTRs must play crucial roles in ToMV multiplication. The 5' UTR shows activity to enhance translational efficiency. The region contains a (CAA) repeat (nts 20–44), to which a host heat-shock protein HSP101 binds, and this binding contributes to the translational enhancement *in vitro* and in yeast (Tanguay and Gallie, 1996; Wells et al., 1998). The 3' UTR of ToMV RNA harbors three consecutive pseudoknots immediately downstream of the termination codon of the CP open reading frame (ORF), followed by a 3'-terminal transfer RNA (tRNA)-like structure. The pseudoknot structure enhances translation in *cis*, and interacts with HSP101 (Tanguay and Gallie, 1996; Wells et al., 1998) and eukaryotic elongation factor 1A (eEF1A) (Zeenko et al., 2002). The tRNA-like structures of tobamoviruses and many other plant viruses interact with various tRNA-modifying enzymes, including aminoacyl-tRNA synthetase and tRNA nucleotidyltransferase (Fechter et al., 2001), but the involvement of these host factors in tobamovirus multiplication is poorly understood.

Plant vacuoles contain high activities of proteases and nucleases that can hamper the biochemical analyses of RNA- and protein-related functions. Indeed, we found that cell extracts prepared from evacuolated plant protoplasts have high translation activity, while those prepared by direct disruption of the original (vacuole-containing) protoplasts do not (Komoda et al., 2004). In this study, we

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searched for proteins that interact with 5' or 3' terminal regions of ToMV genomic RNA from the extracts of evacuolated *Arabidopsis thaliana* protoplasts, which were expected to have low nuclease and protease activities.

Results

Purification of A. thaliana proteins that bind to ToMV RNA

The genomic RNA of ToMV is 6384 nts in length, and harbors 5' and 3' UTRs of 71 nts and 202 nts, respectively (Fig. 1A). To isolate plant proteins that specifically bind to the 5' or 3' terminal regions of ToMV genomic RNA, StreptoTag, an RNA aptamer that binds to streptomycin (Fig. 1B) (Wallace and Schroeder, 1998; Bachler et al., 1999), was utilized. We prepared probe RNAs that carry the StreptoTag and a 5'-terminal sequence (nts 1–277 for st-L5 RNA) or a 3'-terminal sequence (nts 6166–6384 for st-L3 RNA) of ToMV genomic RNA. As a control, st-LR RNA that carries the StreptoTag and an internal region of ToMV RNA (nts 764–1004 within the coding region for the 130K and 180K proteins) was also prepared (Figs. 1A, B).

st-L5, st-L3, and st-LR RNAs were separately incubated with the 30,000 ×*g* supernatant (the S30 fraction) of evacuolated *A. thaliana* protoplast extracts, followed by affinity purification using streptomycin-conjugated Sepharose beads. Analysis of the purified fractions by SDS-PAGE and silver staining revealed that the fractions contained st-L5, st-L3, or st-LR RNAs and their degradation products, as well as putative proteins that were removed by phenol–chloroform extraction (Fig. 1C). The band patterns of putative proteins in the st-L3 and st-L5 RNA-purified fractions were different from each other, and most of these bands were absent in the st-LR RNA-purified fraction (Fig. 1C, asterisks), suggesting that some *A. thaliana* proteins were specifically co-purified with st-L5 or st-L3 RNAs.

Previously, Zeenko et al. demonstrated that eEF1A binds to the 3' terminal region of TMV genomic RNA (Zeenko et al., 2002). The band of approximately 50 kDa in the st-L3-purified fraction (marked by 'X' in Fig. 1C) was identified as eEF1A by immunoblot analysis (Fig. 1D) and by mass spectrometry analysis (MALDI-TOF-MS) of its tryptic digest peptides. This result confirms the results of Zeenko et al. and demonstrates the usefulness of the StreptoTag affinity purification method to isolate ToMV sequence-specific RNA-binding proteins.

Identification of a protein that binds to the 5' terminal region of ToMV genomic RNA

Proteins specifically co-purified with st-L5 or st-L3 RNA were excised from the SDS-PAGE gel, digested with trypsin, and analyzed by mass spectrometry (LC–MS/MS). A MASCOT analysis of the LC–MS/MS data suggested that many of the protein bands represented known or putative RNA-binding proteins. Among these proteins, we focused on a protein found in band 'Y,' which was co-purified with st-L5 RNA (Fig. 1C). The protein showed an approximate molecular mass of 37 kDa on SDS-PAGE, and its 11 tryptic digest peptides showed MS/MS spectra similar to those predicted for an *A. thaliana* protein that contained three KH (heterogenous nuclear ribonucleoprotein K-

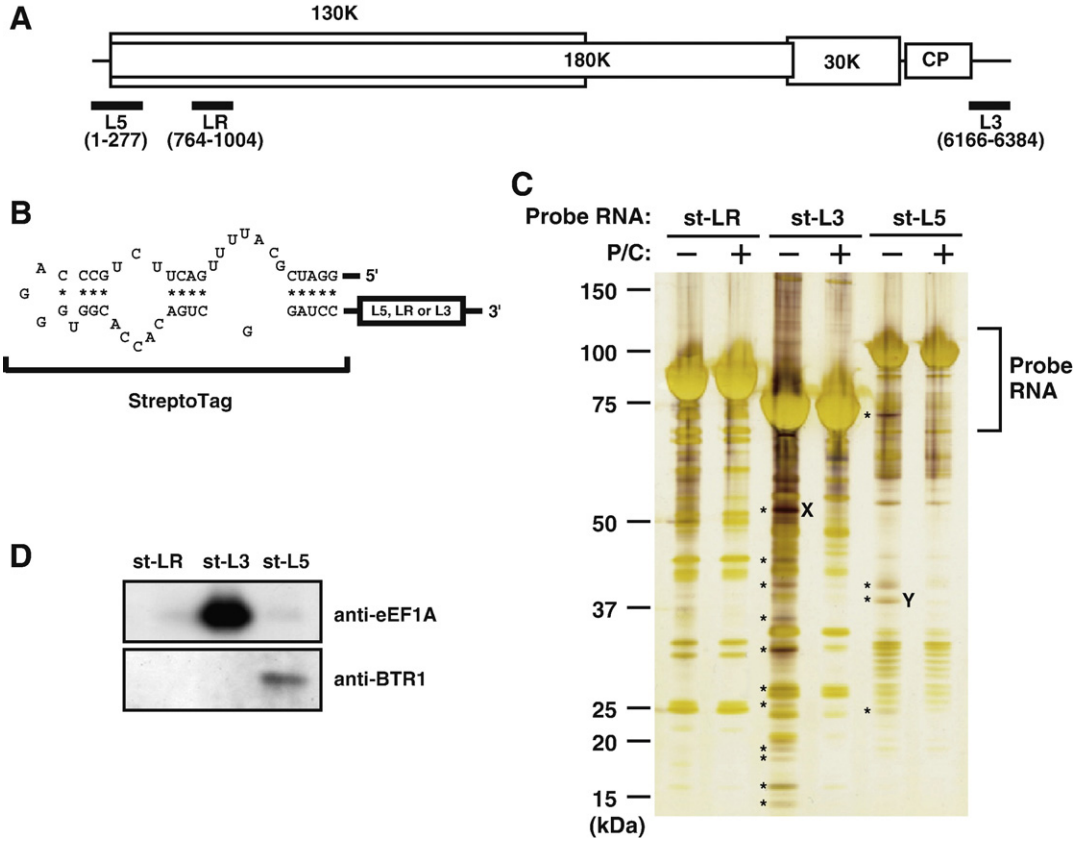

Fig. 1. Purification of *A. thaliana* proteins that bind to ToMV RNA. (A) ToMV genome organization. Thick bars show the regions carried by the probes that were used for protein purification. (B) Structure of the probes. (C) SDS-PAGE patterns of the StreptoTag-purified samples. StreptoTag-purified fractions (for P/C − lanes) were subjected to SDS-PAGE followed by silver staining. The fractions were also extracted with phenol–chloroform, and the aqueous phase was recovered and analyzed in parallel (for P/C + lanes). The positions and molecular masses (kDa) of protein markers are shown on the left side of the gel. Probe RNAs (st-LR, st-L3, and st-L5) used for purification are indicated above the gel. Putative protein bands are denoted by asterisks. The protein bands corresponding to eEF1A and BTR1 are indicated by X and Y, respectively. The positions of the probe RNA bands are shown on the right side of the gel. (D) Specific purification of eEF1A and BTR1 with st-L3 and st-L5 probes, respectively. StreptoTag-purified fractions (P/C-untreated samples in panel C) were subjected to immunoblot analysis using anti-eEF1A or anti-BTR1 antisera.

homology) domains (Fig. 2). The KH domain is known to function in the binding to single-strand nucleic acids in a sequence-specific manner (Auweter et al., 2006), but the function of this specific KH domain-containing protein is not known. We named this protein BTR1 (*B*inding to *T*oMV *R*NA 1). Immunoblot analysis using anti-BTR1 antiserum confirmed st-L5-specific purification of BTR1 (Fig. 1D).

The *A. thaliana* expressed sequence tag database (http://www.arabidopsis.org) suggested that the *BTR1* gene (At5g04430) generates two splicing variant mRNAs that encode different protein products (BTR1L and BTR1S). The BTR1L contains a 21-amino acid region, which is absent in BTR1S, between the second and the third KH domains (Fig. 2). One of the tryptic digest peptide sequences identified by the LC–MS/MS analysis (LTEDDHYSQNVHSPYSYAAGYNSVNYAPNGSGGK) was unique to BTR1S, and a BTR1L-specific peptide sequence corresponding to this region was not detected (Fig. 2), suggesting that the identified protein is BTR1S. In addition, the BTR1 protein from *A. thaliana* leaves showed a similar electrophoretic mobility on SDS-PAGE to that of BTR1S synthesized by *in vitro* translation using a tobacco BY2 cell extract (Fig. 3A). These results suggest that BTR1S is predominantly expressed in *A. thaliana*.

Microarray data (the Bio-Array Resource Database: http://bbc.botany.utoronto.ca/efp/cgi-bin/efpWeb.cgi) suggest that *BTR1* mRNA is ubiquitously expressed in several tissues of *A. thaliana* plants, including rosette leaves, stems, and siliques. The level of BTR1S expression and the BTR1S/BTR1L ratio were not significantly influenced by ToMV infection (Fig. 3A). Consistent with the fact that BTR1S was identified in the S30 fractions of evacuolated protoplast extracts, BTR1S was mainly recovered in the supernatant fraction after 100,000 ×*g* centrifugation (the S100 fraction in Fig. 3B) of *A. thaliana* leaf extracts. These results suggest that, like eEF1A, BTR1S is a soluble protein.

Preferential binding of BTR1S to RNAs carrying a sequence around the initiation codon of the ToMV replication protein gene

We next examined if BTR1S alone can directly bind to the 5' terminal region of ToMV RNA. Deca-histidine-tagged BTR1S protein (His-BTR1S) was expressed in *Escherichia coli*, purified using nickel-nitrilotriacetic acid (Ni-NTA) agarose, and tested by electrophoretic mobility shift assays for the ability to bind selected RNA fragments. When an RNA probe containing the nts 1–300 region of ToMV genomic RNA (ToMV-5; Fig. 4A) was incubated with purified His-BTR1S protein, a complex was formed that resulted in retarded migration of the probe RNA (Fig. 4B). Such complex formation was not observed when ToMV-3 RNA (nts 6166–6384 of ToMV genomic RNA;

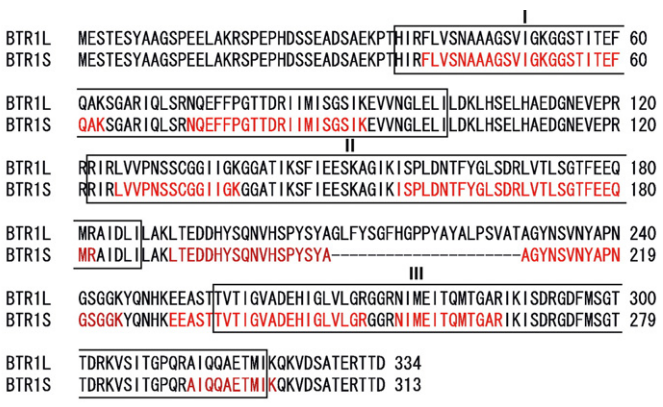

Fig. 2. Alignment of deduced amino acid sequences of BTR1S and BTR1L. The sequence data were obtained from the Arabidopsis Information Resources (http://www.arabidopsis.org). Three KH domains predicted by the Pfam program (http://www.sanger.ac.uk/Software/Pfam/search.shtml) are shown by boxes (I–III). The tryptic digest peptides identified by mass spectrometry (LC–MS/MS) are indicated by red letters.

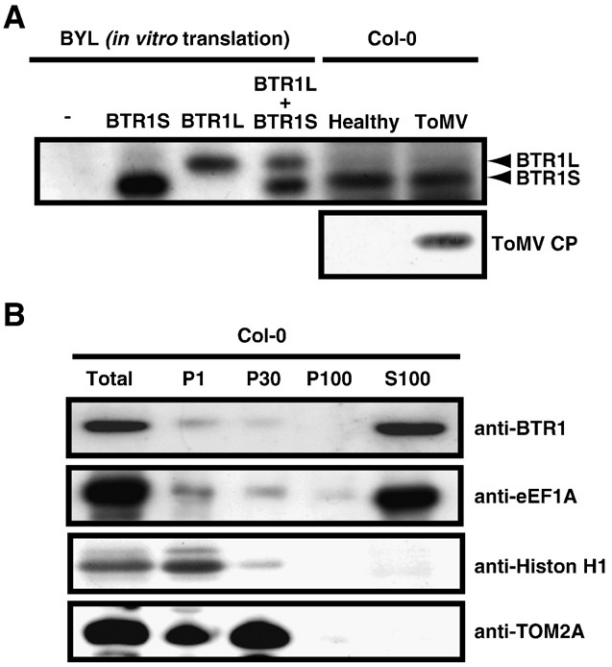

Fig. 3. Expression and subcellular fractionation analyses of the BTR1 protein. (A) Accumulation of the BTR1 protein in *A. thaliana* Col-0 plants. Total proteins from 1 mg (fresh weight) of healthy or ToMV-inoculated (4 dpi) rosette leaves were subjected to SDS-PAGE followed by immunoblot analysis using anti-BTR1 and anti-ToMV antisera (lanes indicated by 'Col-0'). In addition, *A. thaliana* BTR1S and BTR1L mRNAs were translated *in vitro* using BYL (Komoda et al., 2004) and analyzed in parallel (lanes indicated by 'BYL'). A control translation reaction without exogenously added mRNA was also performed (the '–' lane). The positions of BTR1S and BTR1L are indicated on the right side of the gel. (B) Subcellular fractionation of BTR1. Homogenates of rosette leaves of healthy Col-0 plants were subjected to fractionation by differential centrifugation, as described in the Materials and methods. The total protein sample and fractionated protein samples (P1: 1000 × *g* pellet; P30: 30,000 × *g* pellet; P100: 100,000 × *g* pellet; S100: 100,000 × *g* supernatant) were analyzed by SDS-PAGE and immunoblot analysis using antibodies against BTR1, eEF1A (soluble protein), Histone H1 (nuclear protein), and TOM2A (a membrane protein localized mainly on tonoplasts).

Fig. 4A) or an RNA fragment containing a part of the firefly luciferase mRNA sequence (luc) was incubated with the His-BTR1S protein (Fig. 4B), or when ToMV-5 RNA was incubated with the Ni-NTA-purified fraction of the uninduced *E. coli* cell extracts (Fig. 4B, lanes 'U'), or with another purified deca-histidine-tagged polypeptide (data not shown). These results indicate that BTR1S preferentially and directly binds to the 5' terminal region of ToMV genomic RNA.

To map the BTR1-binding site more precisely, three ToMV-5-derived RNAs, ToMV-5a (nts 1–120), ToMV-5b (nts 111–230), and ToMV-5c (nts 180–300) (Fig. 4A), were prepared and tested for the ability to bind His-BTR1S. Among these three fragments, only ToMV-5a RNA showed strong binding to the His-BTR1S protein (Fig. 4C). We then further tested ToMV-5d (the 5' UTR; nts 1–71), ToMV-5e (a region around the initiation codon for the replication proteins; nts 47–120), and ToMV-5f (a coding region for the replication proteins: nts 72–190) (Fig. 4A). Among these fragments, only ToMV-5e RNA showed strong binding to the His-BTR1S protein (Fig. 4D). These results suggest that the nts 47–120 region of ToMV genomic RNA that encompasses the initiation codon for the replication proteins is required for efficient binding to His-BTR1S. This region consists of 22 A, 23 C, 9 G and 20 U residues, and was not predicted to form significant secondary structures (data not shown).

BTR1 negatively regulates ToMV multiplication in A. thaliana plants

To investigate the involvement of BTR1 in ToMV multiplication *in vivo*, we utilized two independent mutant lines of *A. thaliana*

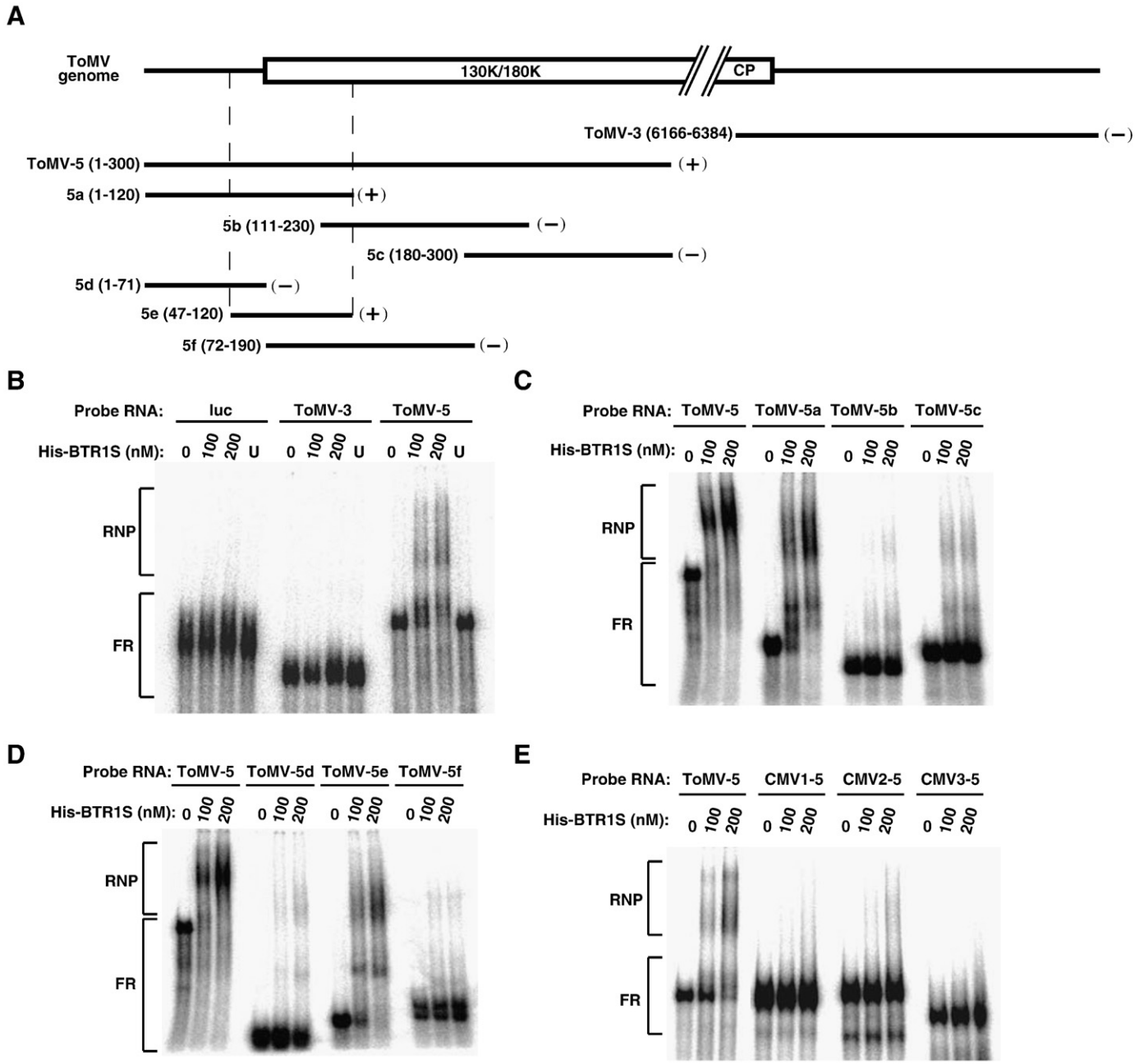

Fig. 4. RNA-binding specificity of BTR1S. (A) Probe RNAs used for electrophoretic mobility shift assay. The probes marked with (+) showed strong binding to the His-BTR1S protein, and those with (−) did not. (B–E) Electrophoretic mobility shift assay. ^{32}P-labeled probe RNAs indicated above the gels were mixed with His-BTR1S (0, 100, or 200 nM) and separated with 4% polyacrylamide-TBE gels. In panel B, RNA carrying part of the firefly luciferase gene (luc), ToMV-3 RNA, and ToMV-5 RNA were used as probes. A Ni-NTA agarose-purified fraction from uninduced *E. coli* cells (lanes 'U') was also used in place of His-BTR1S protein solution. In panel C, ToMV-5, ToMV-5a, ToMV-5b, and ToMV-5c RNAs were used as probes. In panel D, ToMV-5, ToMV-5d, ToMV-5e, and ToMV-5f RNAs were used as probes. In panel E, CMV1–5, CMV2–5, and CMV3–5 RNAs, which correspond to 5' terminal 300 nts sequences of CMV genomic RNA1, RNA2, and RNA3, respectively, and ToMV-5 were used as probes. Positions of free probe RNAs (FR) and BTR1S-RNA complexes (RNP) are indicated on the left side of the gels.

(*btr1-1*: SALK_007924 and *btr1-2*: SALK_047622; Fig. 5A), in which T-DNA fragments were inserted in the *BTR1* gene. Plants homozygously carrying either the *btr1-1* or *btr1-2* mutations did not accumulate the BTR1 proteins to detectable levels (Fig. 5A). ToMV RNA was inoculated to the well-expanded leaves of these plants, and CP accumulation in the inoculated leaves was examined by the immunoblotting method at 4 days postinoculation (dpi), when ToMV CP accumulation was readily detectable but did not reach a plateau in wild-type plants. In *btr1-1* and *btr1-2* mutants, ToMV CP accumulated to higher levels than in wild-type plants (Fig. 5A), suggesting a negative effect of BTR1 on ToMV multiplication.

We then examined the effect of BTR1 overexpression on ToMV multiplication. Wild-type *A. thaliana* (accession Col-0) plants were transformed with gene cassettes containing the BTR1S ORF or BTR1L ORF under the control of cauliflower mosaic virus 35S RNA promoter. BTR1S-overexpressing transgenic (T1) plants including S5 and S12, and BTR1L-overexpressing transgenic (T1) plants including L3 were generated. Progenies of these plants (T2) were inoculated with ToMV, and CP accumulation in inoculated leaves was examined at 4 dpi. In

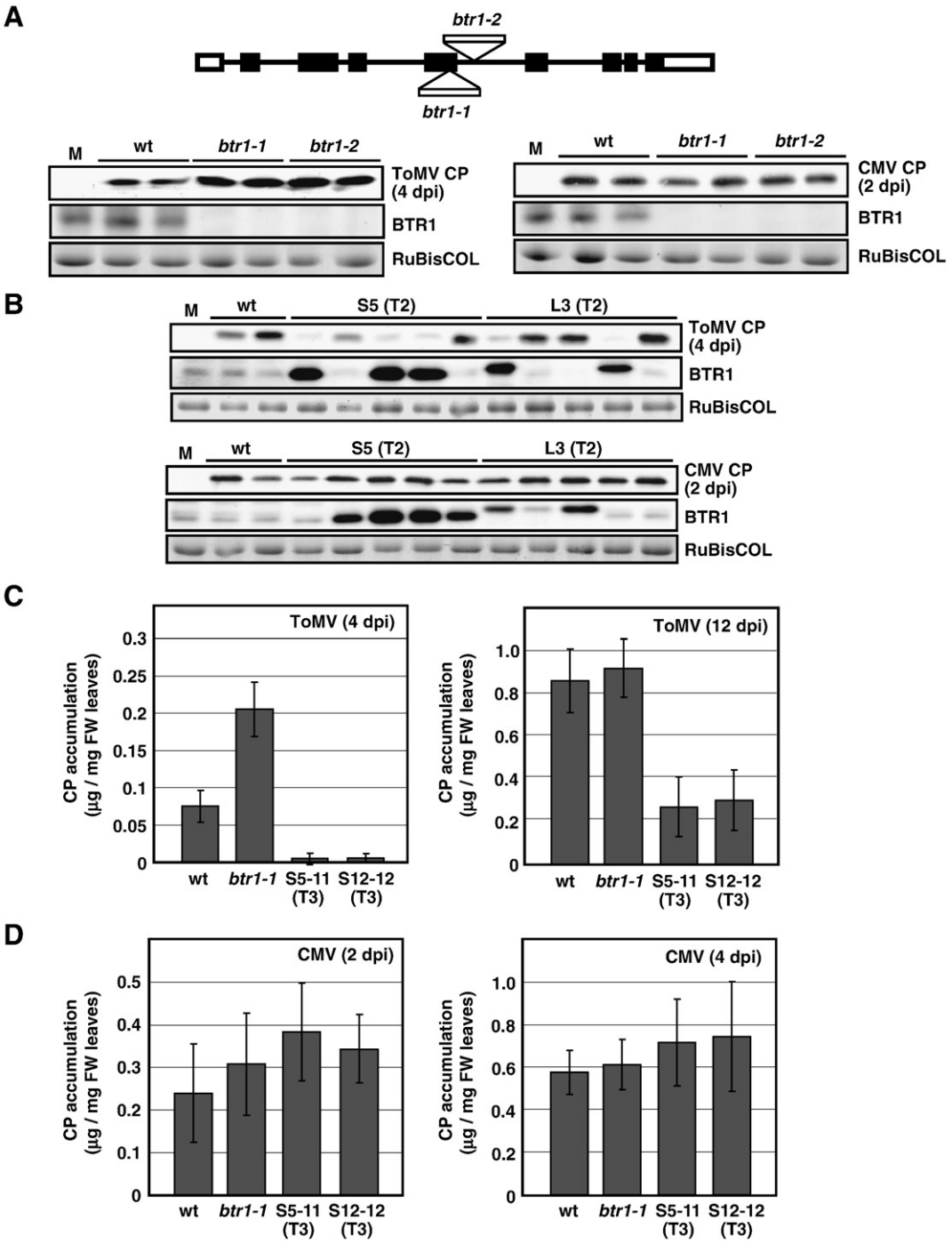

Fig. 5. Effects of knockout and overexpression of BTR1 on viral CP accumulation in *A. thaliana* leaves. (A) ToMV and CMV CP accumulation in *btr1*-knockout plants. Intron–exon organization of the *BTR1* gene is shown at the top. Open and filled boxes indicate noncoding and coding exons, respectively, and introns are denoted by horizontal lines. The positions of T-DNA insertions in the *btr1-1* (SALK_007924) and *btr1-2* (SALK_047622) mutants are also given. The mutant and Col-0 (wt) plants were inoculated with ToMV (left gels) or CMV (right gels); inoculated leaves were harvested at the indicated time points after inoculation, and viral CPs and BTR1 proteins were detected by immunoblotting using specific antisera. A sample derived from an independent plant individual was loaded in each lane. Mock-inoculated Col-0 leaves were analyzed in the lanes marked 'M.' Coomassie brilliant blue-stained bands of ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit (RubisCOL) are shown as a loading control. (B) ToMV and CMV CP accumulation in transgenic plants that overexpressed BTR1S or BTR1L. T2 plants derived from a single T1 plant that overexpressed BTR1S (S5) or those derived from a single T1 plant that overexpressed BTR1L (L3), or Col-0 (wt) plants were inoculated with ToMV (upper three gels) or CMV (lower three gels). Viral CPs, BTR1 and RubisCOL, were detected as described for panel A. Mock-inoculated Col-0 plants (M) were also analyzed in parallel. Consistent results were obtained from four and two independent BTR1S- and BTR1L-overexpressing transgenic lines, respectively. (C) and (D) Quantitative analysis of CP accumulation in plants. The accumulation levels of ToMV (C) and CMV (D) CPs in inoculated leaves of wt, *btr1*-knockout, and BTR1S–overexpressing plants (T3 plants derived from the T2 plants S5-11 and S12-12; see Materials and methods) were quantified by enzyme-linked immunosorbent assay. Means ± standard deviations of CP accumulation levels in eight plants are shown.

the T2 plants that overexpressed BTR1S or BTR1L, ToMV CP accumulation was lower than in wild-type plants or in the T2 segregants that did not overexpress BTR1 (Fig. 5B).

To examine the observed differences more quantitatively, we determined the levels of ToMV CP accumulation in the wild-type Col-0, *btr1-1* (knockout) mutant, and BTR1S-overexpressing plants (T3

plants derived from the T2 plants S5-11 and S12-12; see Materials and methods) using an enzyme-linked immunosorbent assay. At 4 dpi, the accumulation of ToMV CP in *btr1-1* plants and S5-11- and S12-12-derived T3 plants were 273, 8, and 7% of the level in wild-type plants, respectively ($P<0.01$: *t*-test; Fig. 5C). At 12 dpi, the level of ToMV CP accumulation in *btr1-1* mutant plants became similar to that in wild-type plants, while those in S5-11- and S12-12-derived T3 plants were still significantly lower than the wild-type level (approximately 30% of that in wild-type plants; $P<0.01$: *t*-test; Fig. 5C). Consistent with the results with the CP, the accumulation of ToMV-related RNAs in ToMV-inoculated leaves was increased and decreased by knockout of *BTR1* and overexpression of BTR1, respectively (data not shown).

Cucumber mosaic virus (CMV) is a positive-strand RNA virus that belongs to a different taxonomic group from ToMV. Purified His-BTR1S did not detectably bind to the 5' terminal regions of CMV genomic RNAs (Fig. 4E). Well-expanded leaves of *A. thaliana btr1* and BTR1-overexpressing plants were inoculated with CMV RNA, and the multiplication of CMV in the inoculated leaves was examined at 2 dpi, when the average diameter of CMV-positive areas derived from individual infection centers was similar to or slightly larger than that for ToMV at 4 dpi in wild-type *A. thaliana* leaves (data not shown). The accumulation of CMV CP and RNA in *btr1* mutants and BTR1-overexpressing plants was similar to that in wild-type plants (Figs. 5A, B, D and data not shown). The level of CMV CP accumulation was also similar among these genotypes of plants at a later time point (4 dpi: Fig. 5D) These results, together with the fact that the *btr1-1*, *btr1-2*, and BTR1-overexpressing plants grew normally (data not shown), suggest that BTR1 overexpression inhibits ToMV multiplication in a specific manner.

The effect of knockout and overexpression of BTR1S on ToMV multiplication in protoplasts was next examined. Protoplasts were prepared from leaves of wild-type Col-0 and *btr1-1* plants and S5-11- and S12-12-derived T3 plants, and were inoculated with ToMV and CMV RNAs. In *btr1-1* and BTR1S-overexpressing protoplasts, CMV multiplied normally (Fig. 6), confirming that protoplasts of similar quality were prepared. For ToMV, CP and genomic RNA accumulated in *btr1-1* protoplasts to levels similar to those in wild-type protoplasts (Fig. 6). In BTR1S-overexpressing protoplasts, the average levels of ToMV CP and genomic RNA accumulation were 60–80% of wild-type levels although the difference was not significant statistically ($P>0.01$: *t*-test: Fig. 6).

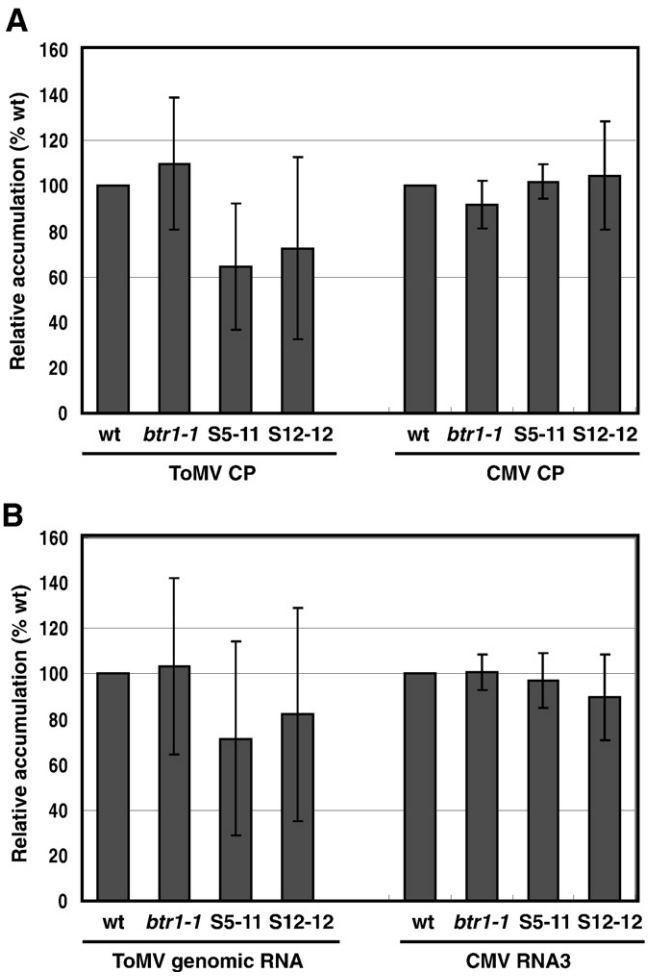

Fig. 6. The effects of BTR1 knockout and overexpression on virus multiplication in protoplasts. (A) The accumulation of ToMV and CMV CPs in *A. thaliana* protoplasts. (B) The accumulation of ToMV genomic RNA and CMV RNA3 in *A. thaliana* protoplasts. Protoplasts derived from wild-type (wt) and *btr1-1* plants and BTR1-overexpressing plants (T3 plants derived from the T2 plants S5-11 and S12-12; see Materials and methods) were inoculated with ToMV and CMV virion RNAs, cultured for 20 h and harvested for analyses. The CPs were detected by the immunoblotting method, and ToMV genomic RNA and CMV RNA3 were detected by Northern blotting and hybridization. The intensity of CP and RNA bands was densitometrically quantified using the Image J program. Values for *btr1-1*, BTR1-overexpressing protoplasts were divided by that for wt protoplasts to obtain relative accumulation levels. Means± standard deviations of the relative accumulation levels of the CPs and indicated RNAs in seven independent experiments are shown.

Overexpression of BTR1 inhibits the translation of BTR1-binding site-containing mRNAs

The 5' terminal regions of mRNAs are often involved in the control of translational efficiency (Dreher and Miller, 2006). To examine if the translational efficiency of BTR1S-binding site-containing mRNAs is affected by the knockout and overexpression of *BTR1S*, capped Fluc and Rluc mRNAs that contained the 5' terminal region (nts 1–278) and the 3' UTR (nts 6183–6384) of ToMV genomic RNA (L278-Fluc and L278-Rluc; Fig. 7A), and control Fluc and Rluc mRNAs that lacked the BTR1S-binding site (L46-Fluc and L46-Rluc; Fig. 7A) were constructed. The L278-Fluc and L46-Rluc mRNAs were mixed together and co-transfected into protoplasts prepared from wild-type and *btr1-1* plants and S5-11- and S12-12-derived T3 plants. After a 3-h incubation, protoplasts were harvested and Fluc and Rluc activities were measured. The Fluc activity relative to the Rluc activity (the Fluc/Rluc ratio) expressed in protoplasts from S5-11- and S12-12-derived T3 plants was approximately 49 and 68%, respectively, that of the wild-type protoplasts ($P<0.01$ by *t*-test; Fig. 7B).

When a similar experiment was performed using L46-Fluc and L278-Rluc mRNAs (Fig. 7A), the Fluc/Rluc ratio observed in protoplasts from S5-11- and S12-12-derived T3 plants was 163 and 178%, respectively, compared to that in wild-type protoplasts ($P<0.01$ by *t*-test; Fig. 7B). These results show the negative effect of BTR1S overexpression on translation of mRNAs containing the BTR1-binding region. In either experiment, the Fluc/Rluc ratio observed in *btr1-1* protoplasts was not significantly different from that in the wild-type protoplasts (Fig. 7B).

The decay rates of L278-Fluc, L46-Rluc, L278-Rluc, and L46-Fluc mRNAs in protoplasts from 0 to 3 h after transfection were not drastically influenced by overexpression of BTR1S (Fig. 7C and data not shown), suggesting that overexpression of BTR1S affects the efficiency of translation rather than mRNA stability.

Discussion

The genomic RNAs of positive-strand RNA viruses encounter numerous interactions with host RNA-binding proteins in infected cells. Such interactions may have positive or negative effects on viral RNA translation, replication, and/or other processes of virus multiplication. In this study, we found that *A. thaliana* BTR1 protein preferentially binds to the 5' terminal region of ToMV genomic RNA.

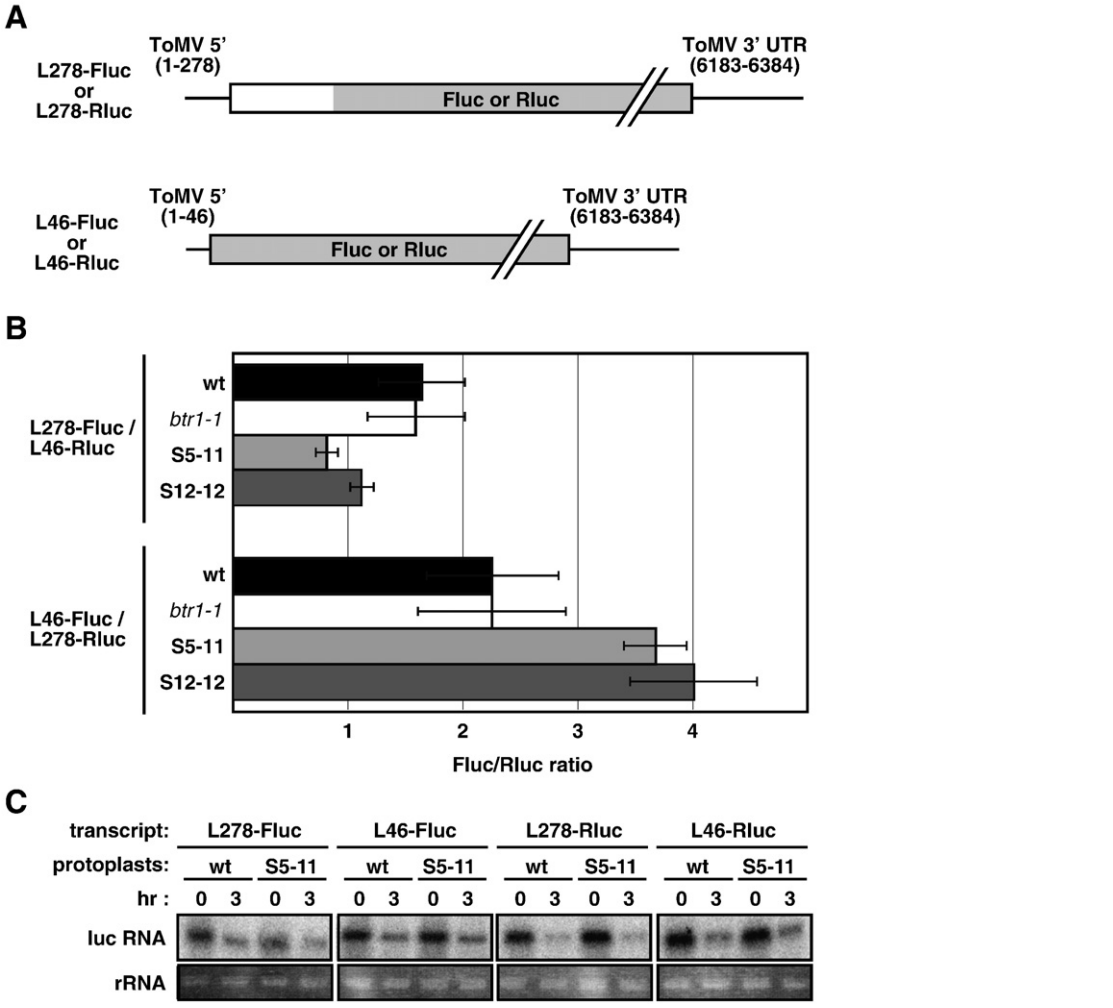

Fig. 7. The effects of *BTR1* knockout and overexpression on the translation of BTR1-binding-site-containing mRNAs. (A) Fluc and Rluc mRNA constructs. Fluc and Rluc coding regions are indicated by gray boxes. 5' and 3' UTRs of ToMV genomic RNA are denoted by horizontal lines, and the coding region of the ToMV 130K/180K gene is indicated by an open box. (B) Luciferase activity expressed in protoplasts. The mixtures of luciferase mRNAs (L278-Fluc plus L46-Rluc or L46-Fluc plus L278-Rluc) were transfected into wild-type (wt), *btr1-1*, and BTR1S-overexpressing protoplasts (from T3 plants derived from the T2 plants S5-11 and S12-12; see Materials and methods), and Fluc and Rluc activities were measured 3 h after transfection. Means ± standard deviations of the Fluc/Rluc activity ratios in four to 11 independent experiments are shown. (C) Stability of Fluc and Rluc mRNAs. L278-Fluc, L46-Fluc, L278-Rluc, or L46-Rluc mRNAs were transfected into protoplasts prepared from wt or S5-11-derived T3 plants, and total RNA was extracted from the protoplasts immediately after and 3 h following transfection. Fluc and Rluc mRNAs was detected by Northern blot hybridization using a ^{32}P-labeled DNA probe specific to the 3' UTR of ToMV RNA. Ribosomal RNA bands visualized by ethidium bromide staining are shown as a loading control (lower panels).

BTR1 consists of 334 amino acid residues and contains three KH domains, which are implicated in single-strand nucleic acid-binding functions (Auweter et al., 2006). Mammalian poly(rC)-binding proteins (PCBPs) have a similar modular structure, consisting of three KH domains, and have some sequence similarity with BTR1 (86 identical and 130 similar amino acid residues when BTR1 and human PCBP2 amino acid sequences were aligned by CLUSTALW). Members of the PCBP family preferentially bind to cytidine-rich RNA elements (Ostareck et al., 1997; Thisted et al., 2001; Wang et al., 1995), and participate in cellular mRNA stabilization and translational and transcriptional controls (Makeyev and Liebhaber, 2002). Human PCBP2 binds to the 5' UTR of poliovirus RNA, and the binding is required for the replication and translation of poliovirus RNA (Blyn et al., 1997; Gamarnik and Andino, 1998). In contrast to the positive roles of PCBP2 in the internal ribosome entry site-dependent poliovirus RNA translation, the binding of PCBP to the coding region of papillomavirus type 16 L2 mRNA and to the 3' UTR of erythroid 15-lipoxygenase mRNA has been reported to cause translational silencing (Collier et al., 1998; Ostareck et al., 1997).

Knockout and overexpression of *BTR1* resulted in an increase and decrease, respectively, of ToMV multiplication in *A. thaliana* leaves. In protoplasts, however, such effect was hardly detectable. These results suggest that BTR1 negatively regulates the local spread of ToMV. Consistent with this possibility, *in situ* detection of ToMV in BTR1S-overexpressing *A. thaliana* leaves showed that the average diameter of ToMV-positive areas derived from individual infection centers was smaller than that in wild-type leaves (Fujisaki and Ishikawa, unpublished result). The mechanism that underlies this negative effect of BTR1 on ToMV spread remains unknown, but there are several possibilities: (i) BTR1 bound to the nts 47–120 (or other regions) of ToMV genomic RNA. The binding of BTR1 to the genomic RNA in the cytoplasm may directly affect the cell-to-cell movement of ToMV through plasmodesmata. As an alternative model, Kawakami et al. (2004) proposed that *Tobacco mosaic virus* spreads cell to cell as intact 'viral replication complexes (VRCs)' that are associated with the endoplasmic reticulum membranes. Thus, it is also possible that BTR1 is recruited to the VRCs with ToMV genomic RNA templates, and negatively regulates cell-to-cell movement of VRCs. (ii) BTR1 overexpression inhibited translation of mRNAs that harbored the BTR1-binding site in the 5' terminal regions more strongly than that of mRNAs that lacked the BTR1-binding site. This result suggests that BTR1 overexpression inhibits ToMV RNA translation thereby causing

reduction in the production of the 130K and 180K replication proteins. The 130K protein plays roles not only in viral RNA replication but also in cell-to-cell movement and suppression of RNA silencing (Hagiwara-Komoda et al., 2008, Hirashima and Watanabe, 2001, Kubota et al., 2003). Therefore, the negative effect of BTR1 overexpression on ToMV multiplication in leaves may be due to inefficient cell-to-cell movement caused by reduced production of the 130K protein. Inefficient suppression of RNA silencing may also contribute to this effect. The knockout of *BTR1* enhanced ToMV CP accumulation in leaves, however, it did not detectably enhance the translation of BTR1-binding site-containing mRNAs. This result argues against the involvement of translation in the negative correlation between BTR1 level and ToMV multiplication, but does not necessarily deny it because, in the *btr1*-knockout plants, the efficiency of BTR1-binding site-containing mRNA translation might be slightly increased, though undetectable in our assay, to cause detectable difference in ToMV CP accumulation in inoculated leaves. (iii) In protoplasts, negative effect of BTR1 on ToMV multiplication was not statistically significant. However, the average levels of ToMV CP and genomic RNA accumulation were slightly decreased by BTR overexpression. If BTR1 weakly affect intracellular multiplication of ToMV, the effect could be enhanced during local spread and become significant when virus multiplication in leaves are examined; because rapidly-multiplying viruses can spread in plants before induced host defense systems become active.

The results obtained in this study shed light on host factors that interact with virus-encoded factors and inhibit virus multiplication. Recently, Zhu et al. screened more than 5000 yeast proteins for those that bind to an RNA motif present in the 3' UTR of BMV RNA that is required for RNA replication, and identified several such proteins. Among these yeast proteins, pseudouridine synthase 4 (Pus4) and actin patch protein 1 inhibited systemic spread of BMV when expressed in *Nicotiana benthamiana* plants. Pus4 also prevented the encapsidation of BMV RNA (Zhu et al., 2007). The *Tm-1* gene of tomato that confers resistance to ToMV was shown to encode a protein that binds to ToMV 130K and 180K replication proteins and inhibits their normal function to replicate ToMV RNA (Ishibashi et al., 2007). These inhibitory factors are derived from non-natural hosts, and it would be presumed that, during adaptation of a virus to a host, the virus evolves to escape from such inhibitory interactions. However, other examples indicate that even a natural host or a host that permit high-level virus multiplication can have inhibitory factors that interact with viral factors (Honda et al., 2007; Paranjape and Harris, 2007). Future studies should clarify to what extent and how prevalently such inhibitory interactions contribute to determine the level of multiplication, virulence, and host specificity of viruses.

Materials and methods

StreptoTag purification

A streptomycin-binding aptamer (StreptoTag: Wallace and Schroeder, 1998; Bachler et al., 1999)-containing transcription vector pStM was constructed by inserting a SacI–XbaI fragment, 5'-*GAGCTC*GGATCGCATTTGGACTTCTGCCCAGGGTGGCACCACGGTCGGATCC**GCATGC**GCGGCCGC**ACGCGT***TCTAGA*-3' [StreptoTag sequence underlined; SacI (*GAGCTC*) and XbaI (*TCTAGA*) sites italicized; SphI (**GCATGC**) and MluI (**ACGCGT**) sites shown in bold letters], between the SacI and XbaI sites of pBluescript II SK(+) (Stratagene). ToMV cDNA fragments containing nts 1–510, 764–1004, and 6166–6384 were inserted between the SphI and MluI sites of pStM to construct pStL5, pStLR, and pStL3, respectively. ToMV genomic (+) RNA probes that carry the StreptoTag at the 5' termini were synthesized with T3 RNA polymerase in the absence of cap structure analogs using EcoRI-linearized pStL5, and MluI-linearized pStLR and pStL3 as templates, and purified using the Mini Quick RNA Spin column (Roche Diagnostics).

The cell extracts of *A. thaliana* were prepared from the suspension-cultured cell line MM2d (Menges and Murray, 2002). Vacuoles of MM2d protoplasts were removed using Percoll density gradient centrifugation as previously described for tobacco BY-2 protoplasts (Komoda et al., 2004). Evacuolated MM2d protoplasts were suspended in a buffer [50 mM Tris–HCl (pH 7.5), 100 mM NaCl, 3 mM $MgCl_2$, 2 mM DTT and 1 tablet of Complete Mini Protease Inhibitor Mixture (Roche Diagnostics) per 10 ml] and disrupted with 100 strokes in a Dounce homogenizer. Resulting cell homogenates were centrifuged at 30,000 ×*g* for 15 min at 4 °C, and the supernatant was used for StreptoTag purification.

StreptoTag purification was performed essentially as described previously (Bachler et al., 1999). Probe RNAs (200 pmol for StL3 and StLR; 500 pmol for StL5) in 20 μl of column buffer [50 mM Tris–HCl (pH 7.5), 100 mM NaCl, 3 mM $MgCl_2$] were denatured at 65 °C for 5 min followed by incubation at room temperature for 5 min, and mixed with 400 μl of evacuolated *A. thaliana* protoplast extracts (protein concentration: 15 mg/ml). The mixture was incubated for 15 min on ice, and then 8 μl of heparin solution (100 mg/ml in column buffer) was added. After further incubation for 30 min on ice, the sample was applied to a column containing 0.8 ml of streptomycin-coupled Sepharose that was pre-equilibrated with column buffer. The column was washed ten times with 2 ml of column buffer, and then the protein–probe RNA complexes were eluted with 3 ml of column buffer containing 10 μM streptomycin. The chromatography was performed at 4 °C. The elution fractions were concentrated 30-fold by acetone precipitation. The elution fractions were also extracted with phenol–chloroform, and the aqueous phase was recovered and concentrated as above. The samples were subjected to SDS-PAGE using a 4–20% Tris-glycine gel (Invitrogen), followed by silver staining. Protein bands were excised, treated with trypsin, and analyzed by mass spectrometry (MALDI-TOF-MS or LC–MS/MS; these parts were performed by the APRO Life Science Institute, Tokushima, Japan).

Characterization of BTR1L and BTR1S

BTR1S and *BTR1L* cDNA fragments were amplified by PCR using primer sets 5'-ACAATTAACCCTCACTAAAGG*GCCAGAGCCACCTTCATTCAC*-3' (the T3 promoter sequence underlined; the 5' UTR sequence of *BTR1* mRNA italicized) and 5'-TTACGCGT*CTCTGCCTCACTCAAAGATG*-3' (the MluI site underlined; the sequence complementary to the 3' UTR of *BTR1* mRNA italicized), and cloned into pCR-BluntII-TOPO (Invitrogen) to obtain plasmids pT3BTR1S and pT3BTR1L, respectively. MluI-linearized pT3BTR1S and pT3BTR1L were used for *in vitro* transcription using an AmpliCap-Max T3 High Yield Message Maker Kit (Epicentre Technologies). Capped transcripts of *BTR1S* (1 μg), *BTR1L* (1 μg), or both (0.5 μg each) were translated in 25 μl of evacuolated tobacco BY2 protoplast lysate (BYL)-based reaction mixtures. Preparation of BYL and *in vitro* translation using BYL were performed as described previously (Komoda et al., 2004).

Antisera

BTR1S cDNA was cloned in pDEST-His (Tsunoda et al., 2005). N-terminally deca-histidine-tagged BTR1S (His-BTR1S) protein was expressed from the plasmid in *E. coli* strain BL21(DE3)pLysS, purified using His Select Nickel Affinity Gel (Sigma), and used to immunize rabbits. Rabbit antisera against ToMV CP, CMV CP, and TOM2A were described previously (Hagiwara et al., 2003; Ishikawa et al., 1993; Saito et al., 1989). Rabbit antiserum against eEF1A was a generous gift from Dr. Karen S. Browning (The University of Texas, Austin). The mouse monoclonal antibody against histone H1 was purchased from Funakoshi Co (Tokyo, Japan).

Electrophoretic mobility shift assay

ToMV cDNA fragments [nts 1–300 (ToMV-5), nts 1–120 (for ToMV-5a), nts 111–230 (for ToMV-5b), nts 180–300 (for ToMV-5c), nts 1–71 (for ToMV-5d), nts 47–120 (for ToMV-5e), nts 72–190 (for ToMV-5f), and nts 6166–6384 (for ToMV-3)] and CMV (strain Pepo: Saitoh et al., 1999) cDNA fragments [nts 1–300 of RNA1 (CMV1–5), RNA2 (CMV2–5), and RNA3 (CMV3–5)] were amplified by PCR using forward primers containing the promoter sequence for T3 RNA polymerase and reverse primers containing the MluI recognition site, and cloned into pCR-BluntII-TOPO. Using these plasmids that had been linearized by MluI as templates, ^{32}P-labeled probe RNAs were synthesized with T3 RNA polymerase and purified by a Mini Quick RNA Spin column (Roche Diagnostics). A probe RNA containing a part of the firefly luciferase (Fluc) coding sequence was synthesized with SP6 RNA polymerase using *Cfr*10I-linearized pSP-luc (Invitrogen) as a template.

The *E. coli*-expressed and purified His-BTR1S protein (0, 2, and 4 pmol) was preincubated in RNA-binding buffer [50 mM Tris–HCl (pH 7.5), 100 mM NaCl, 3 mM $MgCl_2$, 2 mM DTT, 100 mM imidazol, 5% glycerol, and 2 mg/ml heparin] (20 μl) at 30 °C for 10 min. Then, 1 μl of 0.1 μM ^{32}P-labeled probe RNA (0.1 pmol) was added and incubated at 30 °C for 15 min. These samples were mixed with a 2.5 μl of 50% glycerol–0.05% bromophenol blue solution and analyzed by non-denaturing 4% polyacrylamide gel electrophoresis in TBE buffer [45 mM Tris–borate (pH8.3) and 1 mM EDTA]. ^{32}P-labeled RNA bands were detected using a BAS2500 image analyzer (Fujifilm).

Plants and viruses

A. thaliana plants were grown at 22–24 °C with 16 h illumination per day (Fujisaki et al., 2004). The *A. thaliana* T-DNA tag lines [SALK_007924 (*btr1-1*) and SALK_047622 (*btr1-2*)] were obtained from the Arabidopsis Biological Resource Center (http://www.biosci.ohio-state.edu/pcmb/Facilities/abrc/abrchome.htm). The presence of the T-DNA insertions was tested by genomic PCR using three primers: two specific for the *BTR1* genomic sequence (5'-ACGCTTAACCAC-CAAATATC-3' and 5'-ATCCAGCATCTACACTTGAC-3') and one specific for the left border of the T-DNA (5'-CGTGGACCGCTTGCTGCAACT-3'). Plants homozygously carrying the T-DNA insertions were selected, and their progenies were used for the experiments.

To establish BTR1S- and BTR1L-overexpressing plants, *BTR1S* and *BTR1L* cDNAs were amplified by PCR using primers 5'-CTGG*TCTAGA*T-GAGACTTAGTTTTGCTCAG-3' (XbaI site italicized) and 5'-CTGG*GAGCTC*CTCTGCCTCACTCAAAGATG-3' (SacI site italicized); they were then cloned between the XbaI and SacI sites of pBI121 to create the T-DNA clones pBI-BTR1S and pBI-BTR1L, in which the *BTR1S* and *BTR1L* cDNAs were placed under the control of the cauliflower mosaic virus 35*S* RNA promoter. *A. thaliana* plants (accession Col-0) were transformed with pBI-BTR1S and pBI-BTR1L using the *Agrobacterium*-mediated floral dip method (Clough and Bent, 1998). T2 plants derived from a pBI-BTR1S-transformed and BTR1S-overexpressing T1 plant S5, and those derived from a pBI-BTR1L-transformed and BTR1L-overexpressing T1 plant L3 were used for *in planta* virus multiplication assays (Fig. 5B). Quantification of CPs in inoculated leaves (Figs. 5C, D) and protoplast inoculation (transfection) experiments (Figs. 6 and 7) were performed using T3 plants derived from two T2 plants (S5-11 and S12-12; direct descendants of S5 and another BTR1S-overexpressing T1 plant, S12, respectively). All of the T3 plants overexpressed BTR1S as far as we examined, suggesting that S5-11, S12-12, and their progenies carried the transgenes homozygously.

ToMV (strain L) is derived from a full-length ToMV cDNA clone pTLW3 (Kubota et al., 2003), and CMV (strain Pepo) is from pCP1TP1, pCP2TP1, and pCP3TP2 (Saitoh et al., 1999). ToMV was propagated in *Nicotiana tabacum* cv. Samsun, and virions were purified as described previously (Ishikawa et al., 1993). CMV was propagated in *N. tabacum* cv. Samsun NN, and virions were purified as described previously (Takanami, 1981).

Subcellular fractionation of A. thaliana leaf extracts

Rosette leaves were harvested 4 weeks after sowing, and 1 g of leaf tissue was homogenized with 5 ml of TR buffer [30 mM Hepes-KOH (pH 7.4), 80 mM potassium acetate, 2 mM magnesium acetate, 2 mM DTT, 1 tablet per 10 ml of Complete Mini Protease Inhibitor Mixture (Roche Diagnostics)]. Homogenates were filtered through cheesecloth, and the filtrate was centrifuged at 1000 ×*g* for 10 min at 4 °C to obtain the pellet (P1 fraction). The supernatant was recentrifuged at 30,000 ×*g* for 30 min at 4 °C to obtain the pellet (P30 fraction). The supernatant was further fractionated by centrifugation at 100,000 ×*g* for 30 min into the supernatant (S100) and pellet (P100) fractions. P1, P30, and P100 pellets were resuspended in TR buffer to volumes that were the same as those before centrifugation. The fractionated and unfractionated samples of the same volume were analyzed by SDS-PAGE and immunoblotting using specific antibodies and the ECL Plus Western blotting detection system (GE Healthcare Bio-Sciences).

Virus multiplication assays

A. thaliana plants were inoculated with ToMV and CMV, and CP accumulation was examined as described previously (Fujisaki et al., 2006).

Leaf protoplasts of *A. thaliana* were prepared and inoculated with ToMV and CMV RNAs following a protocol obtained from Dr. Jen Sheen's laboratory (http://genetics.mgh.harvard.edu/sheenweb/main_page.html). Briefly, rosette leaves from *A. thaliana* plants of 4–5 weeks after sowing were cut into small pieces and incubated in cellulase solution [1% cellulase R10, 0.1% macerozyme R10, 0.4 M mannitol, 20 mM KCl, 20 mM MES (pH5.7), and 10 mM $CaCl_2$] at room temperature for 2 to 3 h. The solution was filtered through cheesecloth, and the filtrate was centrifuged at 140 ×*g* to collect protoplasts. Protoplasts were washed three times with MMg solution [0.4 M mannitol and 15 mM $MgCl_2$]. Then, 3 μg of virion RNAs (1 μg/μl in distilled water) and 250 μl of PEG solution [40% PEG4000 (w/v), 0.2 M mannitol, 100 mM $CaCl_2$] were mixed with approximately 0.5 to 1.0×10^6 protoplasts in 200 μl of MMg, with gentle swirling. Protoplasts were washed three times with W5 solution [154 mM NaCl, 125 mM $CaCl_2$, 5 mM KCl, 2 mM MES (pH5.7)], and incubated in 5 ml of W5 solution at 23 °C for 2 or 20 h. After incubation, protoplasts were collected and washed with MMg three times. Accumulation of viral CPs and RNAs was analyzed as described previously (Fujisaki et al., 2008).

Transfection of luciferase mRNAs into protoplasts

DNA fragments that consist of the promoter sequence for T3 RNA polymerase, ToMV genomic RNA nts 1–278, Fluc-coding region (from the initiation codon to the termination codon), and ToMV genomic RNA nts 6183–6384 (the 3' UTR) were generated by overlapping PCR and cloned into pCR-BluntII-TOPO to obtain pL278-Fluc. The nucleotide sequences around the 5' and 3' terminus of the ToMV genome were (5'-AA*TTAACCCTCACTAAAGG*GTATTTTTACAACAATTACC-3'; T3 promoter italicized; ToMV genomic sequence underlined) and (5'-GGGCCC*ATATG*AA-3'; ToMV genomic sequence underlined; NdeI site italicized), respectively. A similar construct with the ToMV nts 1–46 sequence in place of the ToMV nts 1–278 sequence was also prepared (pL46-Fluc). In addition, the Fluc-coding regions of pL278-Fluc and pL46-Fluc were exactly replaced by the *Renilla* luciferase (Rluc)-coding region to obtain pL278-Rluc and pL46-Rluc, respectively. NdeI-linearized pL278-Fluc, pL46-Fluc, pL278-Rluc, and pL46-Rluc were used for *in vitro* transcription using AmpliCap-Max T3 High Yield

Message Maker Kit (Epicentre Technologies). Capped transcripts were purified with a Mini Quick RNA Spin column (Roche Diagnostics).

Transcripts were mixed (1 pmol L278-Fluc plus 0.25 pmol L46-Rluc, or 1 pmol L46-Fluc plus 0.25 pmol L278-Rluc) and transfected into 0.5×10^6 protoplasts as described for viral RNA inoculation. At 3 h after transfection, protoplasts were collected, washed with MMg three times, and assayed using the Dual-Luciferase Reporter Assay System (Promega) and a luminometer (DLReady, Turner designs).

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